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Research Note

Enhanced Development of *Cryptosporidium parvum* In Vitro by Removal of Oocyst Toxins from Infected Cell Monolayers

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ABSTRACT: Previous studies have suggested that coccidian oocysts of the genus *Eimeria* contain toxic substances that can inhibit parasite development in cultured cells. To examine whether or not oocysts of *Cryptosporidium parvum* may also contain such toxic substances, Madin-Darby bovine kidney cells were plated into 24-well tissue culture plates in RPMI 1640 medium supplemented with 10% fetal bovine serum and inoculated with CsCl purified oocysts of *C. parvum*. Plates were sealed in candle jars at 37°C so that sporozoites could excyst. After 3 hr, candle jars were opened, and the medium containing excysted and unexcysted oocysts was removed and replaced with fresh medium. ^{3}H-uracil was then added to wells, and plates were reincubated for an additional 65 hr at 37°C in candle jars. Control cultures consisted of infected cultures sealed and incubated in candle jars but not opened, as well as infected cultures exposed to atmospheric oxygen concentrations but not washed free of old medium. Parasite growth was measured as incorporation of [^{3}H]uracil by scintillation counting. Results revealed that washing inoculated monolayers and then adding fresh medium significantly enhanced uracil incorporation.

KEY WORDS: *Cryptosporidium parvum*, Apicomplexa, coccidia, in vitro, cell culture, uracil, candle jar.

Recent studies have suggested that sporozoites of *Cryptosporidium parvum* are frail and must be manipulated minimally following excystation (Woodmansee, 1987; Robertson et al., 1993). Even washing free sporozoites using centrifugation appears to reduce the numbers of viable sporozoites (Woodmansee et al., 1987). Because previous studies had shown that *C. parvum* is capable of excysting at 37°C without the use of traditional excystation conditions such as trypsin and bile salts (Fayer and Leek, 1984; Woodmansee, 1987), Upton et al. (1991) chose to inoculate cell monolayers directly with intact oocysts so that sporozoites would have immediate access to host cells upon excystation. However, inoculating intact monolayers with *C. parvum* oocysts has the potential of adversely affecting the cell monolayer. Several studies have shown the presence of toxic substances within coccidial oocysts that might affect development (Burns, 1959; Rickimaru et al., 1961; Sharma and Foster, 1964; Patton, 1965; Fayer and Hammond, 1967; Doran, 1970). Sharma and Foster (1964) found that sporozoites and oocyst and sporocyst walls of *Eimeria tenella* were nonlethal for rabbits following intravenous inoculation whereas fluids in oocysts were highly toxic in vivo. Fayer and Hammond (1967) found that fluid extracts from *Eimeria bovis* oocysts were toxic to bovine cells in vitro. Patton (1965) suggested that oocyst and sporocyst debris were toxic to cell monolayers infected with *E. tenella*.

The preceding studies collectively suggest that although inoculating monolayers directly with oocysts of *C. parvum* may be an effective method of establishing infections in vitro, further studies are needed to determine whether or not toxic substances liberated by this parasite upon excystation have a negative impact on parasite development. Below we present results that demonstrate the adverse effect of toxic substances associated with *C. parvum* oocysts on development of this parasite in cultured cells.

Cryptosporidium parvum was passaged through 2–5-day-old goats (*Capra hircus*) as described previously (Tilley and Upton, 1990). Oocysts were partially purified using discontinuous sucrose gradients (Arrowood and Sterling, 1987) and then further purified using CsCl gradients (Taghi-Kilani and Sekla, 1987). Oocysts purified by CsCl gradient purification were resuspended in 10% (v/v) aqueous Clorox® bleach for 10 min on ice. Oocysts were then washed 3 times with ice-cold, sterile distilled water and 2 times with ice-cold, sterile phosphate-buffered saline (PBS) (pH 7.2). At the time when oocysts were added to the cells, a small aliquot in the experimental medium was removed, placed in a separate test tube, and incubated at 37°C for 60 min. After 1 hr, the sample was viewed under ×40, and the first 100 oocysts observed were counted to determine percentage of excystation. Aliquots wherein <50% of the oocysts were excysted after

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1 hr were deemed unsuitable for in vitro studies, and the experiments were terminated.

The cell culture medium consisted of RPMI 1640 with L-glutamine, supplemented with 0.02 g/ml sodium bicarbonate, 10 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. For routine cell passage, 5% fetal bovine serum (FBS) was used, whereas experiments with parasites employed 10% FBS.

Madin-Darby bovine kidney cells (ATCC #6071) were maintained in 75 cm^2 tissue culture flasks. A trypsin/ethylenediamine tetraacetic acid (EDTA) (trypsin 1:25/0.53 mM EDTA in PBS) solution was then added to lift the cells off the plate. Cells were routinely tested for contamination with species of *Mycoplasma* using 4′,6-diamidino-2-phenylindole stain (Russell et al., 1975; Mitchell and Finch, 1977; Uphoff et al., 1992).

Four hours prior to inoculation with *C. parvum,* cells were plated into 24-well cluster plates at a concentration of 1.5 × 10^5 viable cells in 0.5 ml medium. Preliminary experiments showed that this concentration of cells allowed near-confluent monolayers to be achieved after about 68 hr postinoculation. Cell viability was assessed using trypan blue exclusion (0.15% w/v in PBS), and numbers were quantitated using a hemacytometer. Plates were incubated in a 37°C humidified incubator supplemented with 5% CO_2 until inoculated with oocysts.

Oocysts were added to tissue culture wells in ½ final volume of medium with 15% FBS, bringing the FBS concentration in all wells to 10%. Various concentrations of oocysts were examined, ranging from 1.75–6.25 × 10^5/well (Fig. 1). The plates were then placed in prewarmed desiccator jars (candle jars), a candle inside was lit, and the jars were sealed with stopcock grease. After 3 hr at 37°C, plates were removed from some of the candle jars, the medium removed from some of these, and fresh medium with 10% FBS added. [^{3}H]uracil was also added to wells at this time at a concentration of 4 μCi/well (specific activity 37.7 Ci/mmol). Previous studies have shown these parasites to incorporate free uracil (Upton et al., 1991). Plates removed from candle jars were then placed back in candle jars, resealed following relighting of the candles, and incubated at 37°C for an additional 65 h. Control cultures consisted of infected and noninfected wells without [^{3}H]uracil, cultures incubated within candle jars without breaking the seal, and cultures incubated in candle jars, exposed to atmospheric conditions, and then resealed in candle jars without a change of medium. For all experiments, tissue culture plates were examined immediately upon removal from candle jars by using an inverted microscope at ×20–40. Any well detected to have either bacterial or fungal contamination, or sloughing of the monolayer greater than what appeared to be 25% of the total cells, was eliminated from further consideration. Cells were then harvested by vacuum aspiration onto 0.45-μm pore size glass fiber filters using an Inotech cell harvester (Inotech Biosystems International, Lansing, Michigan). Medium was removed by aspiration, and each well was washed 2 times with distilled water. To each well, 100 μl of 10% (w/v) trichloroacetic acid (TCA) (5 g/50 ml) was added, maintaining vacuum to prevent backwash. After 8–10 min, the TCA was then removed and wells washed 2 additional times with distilled water. To each well, 0.2 M NaOH was then added at a concentration of 400 μl/well, again maintaining vacuum. After 5 min, plates were then washed and aspirated onto filters 3 times with distilled water, followed by 2 washes with 70% EtOH. Filters were detached from the

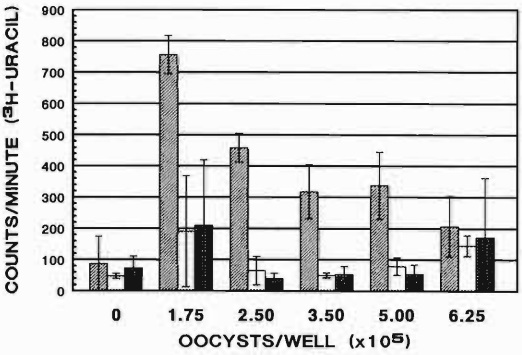

Figure 1. Effects of washing Madin-Darby bovine kidney cells 3 hr postinoculation (PI) with oocysts of *Cryptosporidium parvum.* Diagonal lines represent wells washed 3 hr PI; stippling represents those wells placed under same conditions as washed wells, removed from candle jar and incubator, placed at room temperature for 45 min, and then returned to the jar without replacing media. Cross-hatching represents those unwashed wells left in a second sealed candle jar at 37°C during the entire procedure. Experiments were performed in 24-well plates with 1 ml medium and 4 μCi [^{3}H]uracil per well. Cells were plated 4 hr preinoculation at a concentration of 1.5 × 10^5 per well. Incubation was at 37°C in a sealed candle jar for 65 hr postwashing. All data points represent the mean ± SD of 4–6 replicates.

harvester and placed in a 37°C incubator until dry. Once dried, the filter discs corresponding to each well were placed in 5-ml plastic scintillation vials with 2 ml scintillation cocktail for counting in a Beckman LS 6000SC scintillation counter.

Four to 6 wells were employed for each variable. Data are presented as the mean of 4–6 wells ± SD of the mean. Individual data points were compared among and between groups using a 2-tailed Mann-Whitney *U*-test. In addition, all 3 treatments for each oocyst concentration were compared using the Kruskal-Wallis test. Differences were considered significant for both tests when $P \leq 0.05$.

Two of 5 (40%) experiments resulted in enough development beyond uninfected control cultures to be termed successful. All unlabeled control cultures had tritium counts <20 counts per minute and are not shown in Figure 1. These studies suggested that washing empty and unexcysted oocysts from the monolayers, as well as fluids released from oocysts during excystation, has a positive effect on [^{3}H]uracil incorporation (Fig. 1). After comparison using both tests, it was determined that washing significantly increased uracil incorporation when all oocyst concentrations were employed except 6.25×10^5 ($P < 0.05$). Thus, it appears that debris and/or oocyst fluid from excystation does have a negative effect on in vitro quantitation of *C. parvum.*

Multiple concentrations of oocysts were added to wells for each experiment. Results of these and additional studies in our laboratory have suggested that an oocyst-to-host cell ratio of about 1:1 to 1:2 results consistently in highest uracil incorporation. However, these numbers are highly variable as the percentages of oocysts that excyst within culture vary considerably among experiments. It should be noted that Rasmussen et al. (1993) also found that better parasite development can be achieved with low parasite-to-host cell ratios.

Many studies have collectively demonstrated *Cryptosporidium parvum* to be capable of completing at least some development in a variety of cultured cells (Woodmansee and Pohlenz, 1983; Current and Haynes, 1984; Naciri et al., 1986; Wagner and Prabhu Das, 1986; Lumb et al., 1988; Datry et al., 1989; Bonnin et al., 1990; McDonald et al., 1990; Aji et al., 1991; Buraud et al., 1991; Flanigan et al., 1991; Gut et al., 1991; Kuhls et al., 1991; Upton et al., 1991; Marshall and Flanigan, 1992; Martinez et al., 1992; Rasmussen et al., 1993; Rosales et al.. 1993). Although these studies have helped define some parameters that enhance development of this parasite in vitro, additional studies are needed to provide more reliable, reproducible, and cost-effective in vitro systems that will allow for both studying the basic biology of the parasite and for large-scale pharmaceutical testing.

This work is a portion of a thesis entitled "Development of a ^{3}H-Uracil Incorporation Assay to Monitor Development of *Cryptosporidium parvum,*" submitted by M.T.E. in partial fulfillment of the requirement for a Master of Science degree in Biology, Kansas State University. This research was supported by NIH grant AI31774 to S.J.U. and is Kansas Agricultural Experiment Station contribution No. 93-374-J.

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